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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 20 February 1995 in connection with Application No. PN 1239 for a patent by THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH filed on 20 February 1995.

I further certify that the annexed documents are not, as yet, open to public inspection.

WITNESS my hand this Twenty-seventh day of February 1996

DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

Regulation 3.2

THE WALTER AND ELIZA HALL
INSTITUTE OF MEDICAL
RESEARCH

A U S T R A L I A
Patents Act 1990

PROVISIONAL SPECIFICATION
for the invention entitled:

"IMMUNOINTERACTIVE MOLECULES"

The invention is described in the following statement:

IMMUNOINTERACTIVE MOLECULES

The present invention relates generally to molecules such as peptides, polypeptides and proteins which interact immunologically with T-lymphocytes in subjects having pre-clinical or clinical Insulin-Dependent Diabetes Mellitus (IDDM). These molecules are preferentially immunointeractive to T-cells in subjects having preclinical or clinical IDDM and are useful in the development of diagnostic, therapeutic and prophylactic agents for IDDM.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

IDDM results from the destruction of the insulin-secreting β cells, probably mediated by T cells that recognise β-cell autoantigens. A major antigen implicated in T-cell destruction characteristic of IDDM is glutamic acid decarboxylase (GAD), which occurs in two major isoforms, GAD 65 and GAD 67. These two isoforms share 65% homology. Subjects with IDDM or at high-risk of the disease show antibody and T-cell responses to GAD and/or insulin. In NOD mice, an animal model of spontaneous IDDM, GAD is a dominant and early target antigen (Tisch et al (1993) Nature 366:p72-75).

Identification of the immunodominant epitope(s) of pathogenic autoantigens, HLA molecules and T-cell receptor(s) involved in this β-cell autoimmunity could lead to improved methods of diagnosis as well as therapeutic strategies to prevent IDDM.

In work leading up to the present invention, the inventors sought to identify immunodominant epitopes, in GAD and proinsulin molecules, in order to improve upon current diagnostic procedures and to further develop therapeutic and prophylactic compositions and treatment approaches for IDDM.

In accordance with the present invention, peptides, were synthesised based on a thirteen amino acid region of high similarity between the sequences of human GAD 65 (506-518) and human proinsulin (24-36), which region of similarity also extended to human GAD 67 and both mouse proinsulin and GADs (Figure 1). The immunointeractivity of these molecules is identified on the basis of interactivity of T-cells obtained from subjects suffering from preclinical or clinical IDDM, thereby forming the basis for a new range of diagnostic, therapeutic and prophylactic procedures for IDDM.

Accordingly, one aspect of the present invention provides recombinant or synthetic peptide molecules of the formula $[X_1]_{n1} - [X_2]_{n2} - [X_3]_{n3}$ wherein:
X1 and X3 may be the same or different and each is a natural or non-naturally occurring amino acid;
X2 is any amino acid sequence derived from, homologous to or contiguous within amino acid sequences of human GAD 65 (506-518) and/or human proinsulin (24-36) including additions, deletions and/or substitutions therein or other derivatives within this region;
n1 and n3 may be the same or different and each is from 0-40;
n2 is not less than about 10 and may be up to about 100; and
wherein said peptides cause T-cell activation when incubated with peripheral blood mononuclear cells (PBMC) obtained from patients having IDDM or who are considered to have preclinical IDDM.

In a preferred embodiment n2 is not less than about 10 and not greater than about 50, more preferably n2 is not less than about 10 and not greater than about 30 and even more preferably n2 is not less than about 10 and not greater than about 15.

In a particularly preferred embodiment X1, X3, n1 and n3 are as described above and X2 has either of the following amino acid sequences:

F F Y T P K T R R E A E D; or

F W Y I P P S L R T L E D.

The peptides including polypeptides of the present invention may, for example, be prepared by recombinant means. According to this aspect of the present invention, there is provided a recombinant peptide or polypeptide which is preferentially immunologically reactive with T-cells from individuals with clinical or preclinical IDDM, which is prepared by the expression of a host cell transformed with a cassette coding for peptide sequences described above. The peptide or polypeptide may be fused to another peptide or polypeptide. Alternatively, it may be prepared by chemical synthesis, such as by the well-known Merrifield solid-phase synthesis procedure. The synthetic or recombinant peptide or polypeptide may or may not retain GAD activity or proinsulin activity. Furthermore, although synthetic peptides of the formula above represent a preferred embodiment, the present invention also extends to biologically pure preparations of the naturally occurring peptide or peptides or fragments thereof. By "biologically pure" is meant a preparation of at 60%, preferably at least 70%, more preferably at least 80% and still more preferably at least 90% by weight of peptide.

By "derivatives" is meant to include any single or multiple amino acid substitution, deletion and/or addition relative to the preferred peptide sequence and including any single or multiple substitution, deletion and/or addition to other molecules associated with the peptide or polypeptide including carbohydrate lipid and/or other proteinacious moieties and includes the substitution by any non-naturally occurring amino acids. Such derivatives, therefore, include glycosylated or non-glycosylated forms or molecules with altered glyclosylation patterns.

The invention also extends to use of the peptides and/or polypeptides, or fragments, or derivatives of the present invention in the treatment of patients. In this later aspect, such methods of treatment include their use as an adsorbent to remove autoantibodies or

autoreactive cells from a patient, their use in direct administration to a patient as a means of desensitising or inducing immunological tolerance or other mechanisms to eliminate or diminish reactivity of autoreactive T-cells or autoantibodies to the IDDM autoantigen or to generate T-cell lines or clones to be used for or as therapeutic agents.

As contemplated herein, the method of treatment includes but is not limited to the following examples of treatment. A first example of treatment is desensitisation or tolerance induction use an effective amount of synthetic peptide or polypeptide or fragments thereof to alter T-cell recognition of GAD and/or pro-insulin and/or induce T-cell suppression. This may be achieved by using the known effect of certain ultraviolet wavelengths, especially UV-B, to modify antigen presentation through the skin (see Ullrich et al (1986) Immunology 58, 158-90). Effective amounts of peptides or polypeptide or fragments thereof would be applied epicutaneously to the skin of subjects exhibiting peripheral blood T-cell reactivity to peptides or polypeptides. After exposure of skin to UV-B radiation, treatment would be repeated until such time that T-cell reactivity to GAD was suppressed. A second treatment involves application of peptides or polypeptides to the skin together with one or more cytokines such as but not limited to TNFα or β. A third treatment involves T-cell immunisation whereby T-cell lines are generated to GAD peptide or polypeptide or fragments thereof by standard procedures, cells attenuated by fixation with agents such as glutaraldehyde or paraformaldehyde, washed under sterile conditions and re-injected to patients for a time and under conditions causing suppression of the endogenous T-cell response to autoantigens. These approaches to treatment are applicable to the prevention of clinical IDDM in asymptomatic subjects with preclinical IDDM or subjects with recent onset clinical IDDM, as well as to the recurrence of IDDM in subjects who have received pancreas, islet cell or insulin-producing cell transplants. These approaches are also applicable to Stiff man Syndrome (SMS) and other diseases where GAD and/or insulin is an autoantigen. In accordance with the present invention the effective amount of peptide or polypeptide is 0.1 μg to 10 mg per dose and preferably 1.0 μg to 1 mg per dose. A dose may comprise s single administration or an administration protocol. Administration may be by any convenient means such as, but not limited to, intravenous, subcutaneous, epicutaneous, infusion, oral, topical, intranansal, suppository or

intraperitoneal administration. The peptide or polypeptide may be administered alone or in combination with one or more other active molecules, molecules which facilitate the peptide or polypeptide activity such a cytokines, and in particular, TNF-α and/or TNF-β.

In yet a further embodiment, the present invention contemplates the use of the synthetic peptide or polypeptide to measure reactivity of a subject's cells to the IDDM autoantigen. The peptide or polypeptide, or fragments or derivatives thereof, may be added, in solution or bound to a solid support together with cells from a subject derived from peripheral blood or from tissue biopsies either unfractionated, fractionated or derived as a continuous cell lines. Reactivity to the autoantigen may then be measured by standard proliferation assays such as incorporation of tritiated thymidine, standard cytotoxic assays such as release of marker radioactivity from target cells, measurements of expressed or secreted molecules such as cytokines or other standard assays of cellular reactivity which are well known in the art.

In one embodiment of this aspect of this invention there is provided a diagnostic kit for assaying T-cells. Standard 96 - well plates, as used in ELISA assays, are pre-coated with a monoclonal antibody (MAb) to a T-cell cytokine such as γ-interferon (γ-IFN) with or without antigen. Alternatively, antigen is added in soluble form together with aliquots of peripheral blood mononuclear cells or T-cells. Incubation is allowed to proceed for two or more days, the cells are washed off, wells washed again and plates developed with a labelled second MAb to the cytokine such as anti-γ-IFN conjugated with alkaline phosphatase or horseradish peroxidase. Colorimetric reaction and read-out systems can then by utilised. Alternatively, it is possible to visualise microscopically individual spots on bottoms of wells representing cytokine produced at the single T-cell level, thereby enabling the precursor frequency or antigen-reactive T-cells to be determined.

The present invention will now be further described with reference to the following non-limiting Figures and Examples.

In the Figures:

Figure 1 shows a comparison of the regions of similarity among mouse and human proinsulins and GADs. Similarities are boxed; idealities within boxes are shaded. The C-terminus of the insulin B-chain and the cleavage site of PC3 are indicated by the vertical line and arrow respectively.

EXAMPLE 1

T-cell proliferation assay

Peripheral blood mononuclear cells are isolated from heparinized whole blood by Ficoll-Paque (Pharmacia Biotech) density centrifugation, washed and resuspended in RPMI 1640 medium (Biosciences Pty Ltd) containing 200mM Hepes (CSL Ltd), 10^{-5} M 2-mercaptoethanol (BDH), penicillin (100U/ml), streptomycin (100μg/ml), and 10% autologous plasma. Aliquots of 200μl ($4x10^5$ cells) are transferred into wells of a 96-well, round-bottomed plate (Falcon) and incubated in replicates of six with the following peptides at final concentrations of 10.2 and 0.4μg/ml: human GAD65 (506-518), human proinsulin (24-36) (synthesised using an Applied Biosystems Model 431A Synthesiser (ABI, Foster city, CA)), and an irrelevant control (CRFDPQFALTNIAVRK) (Macromolecular Resources, Fort Collins, CO). Tetanus toxoid (CSL Ltd) at final concentrations of 1.8, 0.18 and 0.018 LfU/ml are used as a positive control, and twelve "autologous only" wells containing unstimulated cells are included as a negative control. Plates are incubated at 37°C in a 5% CO_2 humidified incubator for 6 days; 0.2mCi of [^{3}H]thymidine (ICN) is added to each well for the last 6 hours. The cells are then harvested onto glass fiber filters and incorporated radioactivity measured by beta-particle counting (Packard Model 2000 Liquid Scintillation Counter). Cellular proliferation is expressed as the stimulation index (SI=median counts per minute (cpm) incorporated in the presence of antigen, divided by the median cpm of the "autologous only" wells). A positive result is defined as a stimulation index greater than that obtained with irrelevant or negative control.

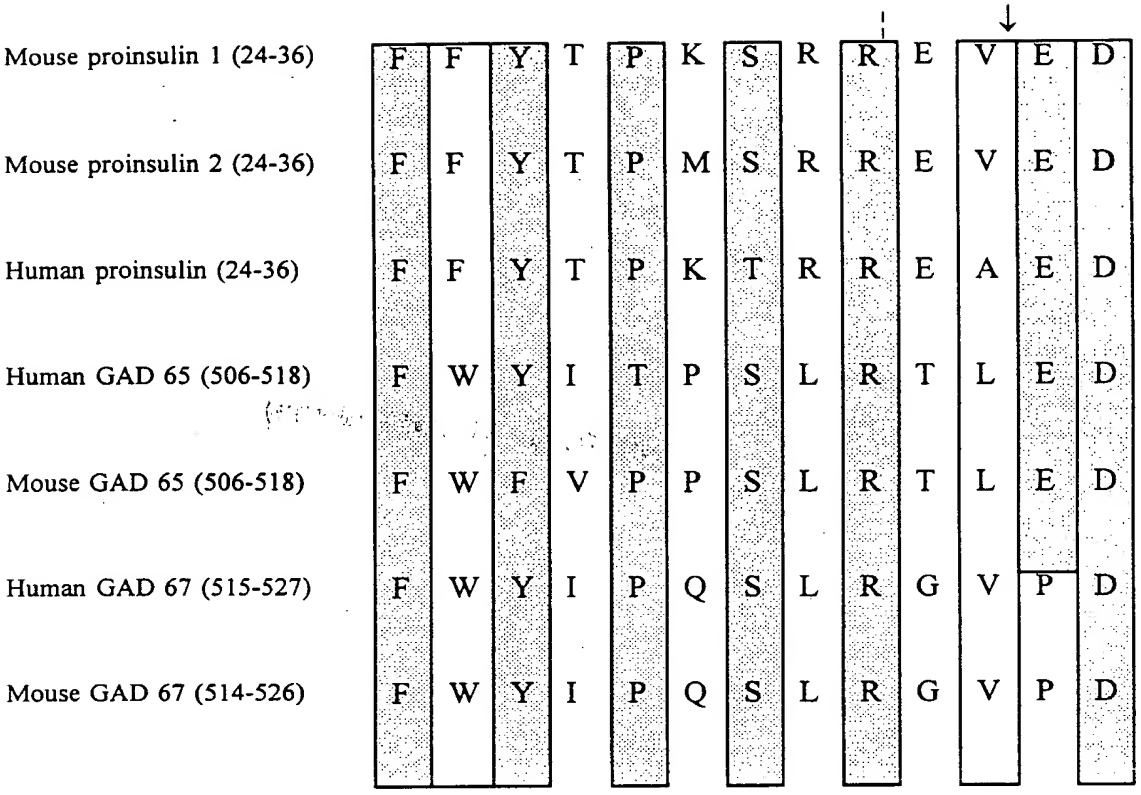
Patients from which blood samples are taken include the following groups;
Preclinical IDDM patients who are identified from screening studies of first degree relatives of diabetic patients performed at the Royal Melbourne Hospital.
Recent Onset Clinical IDDM patients
Control patients who are healthy volunteers and unrelated to diabetics
Control patients who are been diagnosed as suffering from another autoimmune disease than IDDM.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to our indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

DATED this 20th day of February, 1995

THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH
By Its Patent Attorneys
DAVIES COLLISON CAVE

Figure 1



Region of similarity among mouse and human proinsulin and GADs. Similarities are boxed; identities within boxes are shaded. The C-terminus of the insulin B-chain and the cleavage site of PC3 are indicated by the vertical line and arrow, respectively.